

Identification and Analysis of ADP-Ribosylated Proteins

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Abstract The analysis of ADP-ribosylated proteins is a challenging task, on the one hand because of the diversity of the target proteins and the modification sites, on the other hand because of the particular problems posed by the analysis of ADP-ribosylated peptides. ADP-ribosylated proteins can be detected in in vitro experiments after the incorporation of radioactively labeled or chemically modified ADP-ribose. Endogenously ADP-ribosylated proteins may be detected and enriched by antibodies directed against the ADP-ribosyl moiety or by ADP-ribosyl binding macro domains. The determination of the exact attachment site of the modification, which is a prerequisite for the understanding of the specificity of the various ADP-ribosyl transferases and the structural consequences of ADP-ribosylation, necessitates the proteolytic cleavage of the proteins. The resulting peptides can afterwards be enriched either by IMAC (using the affinity of the pyrophosphate group for heavy metal ions) or by immobilized boronic acid beads (using the affinity of the vicinal ribose hydroxy groups for boronic acid). The identification of the modified peptides usually requires tandem mass spectrometric measurements. Problems that hamper the mass spectrometric analysis by collision-induced decay (CID) can be circumvented either by the application of different fragmentation techniques (electron transfer or electron capture dissociation; ETD or ECD) or by enzymatic cleavage of the ADP-ribosyl group to ribosyl-phosphate.

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Abbreviations

ADP	Adenosine diphosphate
CID	Collision-induced decay
IMAC	Immobilized metal ion affinity chromatography
ECD	Electron capture dissociation
ETD	Electron transfer dissociation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HNP-1	Human neutrophil peptide-1
LC/MS	Liquid chromatography/mass spectrometry
MS/MS	Tandem mass spectrometry
NAD	Nicotinamide adenine dinucleotide
QTOF	Quadrupol time-of-flight
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

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1 Introduction

The analysis of any post-translational modification crucially depends upon the methods available for the detection of modified substrates. The requirement profile for an ideal method for the detection of a post-translational modification is complex, and cannot be fulfilled by any single technique. An ideal method should be

sensitive and specific, allow the visualization of modified proteins both within analytical procedures such as SDS-PAGE or gel chromatography as well as within their native cellular environment, and give information about the number and intramolecular location of the attached modifications.

The term ADP-ribosylation describes the transfer of an ADP-ribose moiety from NAD⁺ to a target structure under the release of nicotinamide. Although this review focuses on the analysis of ADP-ribosylated proteins, it is important to bear in mind that other structures such as DNA, RNA, antibiotics, and water may also serve as acceptors of ADP-ribose. Since its discovery in the late 1960s, a variety of methods have been developed to visualize the incorporation of ADP-ribose into proteins, including the introduction of chemical modifications into the substrate NAD⁺, the development of antibodies and natural ligands to detect the modification, and the refinement of mass spectrometry techniques to identify ADP-ribosylated proteins and to determine the modification site.

However, in comparison to phosphorylation, another post-translational modification that profoundly impacts target protein function, the development of methods to identify and analyze ADP-ribosylated substrates lags far behind. Two factors have greatly helped to analyze the role of phosphorylation in signal transduction pathways that are not available in a comparable manner for ADP-ribosylation: the development of algorithms to define consensus sequence motifs that allow the prediction of potential phosphorylation sites (He et al. 2011; Ubersax and Ferrell 2007), and the increased availability of phosphorylation site-specific antibodies that are able to directly report the presence or absence of a phosphorus residue at a specific site within a protein substrate (Macek et al. 2009). Importantly, the availability of site-specific antibodies permits the identification and analysis of endogenously phosphorylated proteins, whereas the field of ADP-ribosylation is still largely dependent on analyzing the products of ADP-ribosylation reactions performed *in vitro* using chemically modified versions of the substrate NAD⁺.

A further complication in the analysis of ADP-ribosylated proteins is the heterogeneity of ADP-ribosylation reactions. ADP-ribosyltransferases have been divided into the two families of ARTC (C2/C3 toxin-like) and ARTD (diphtheria toxin-like) enzymes. While all known poly-ADP-ribosyltransferases belong to the ARTD family, and mono-ADP-ribosylation has traditionally been the domain of the ARTC transferases, it has recently been appreciated that some ARTD family members and even some family members of the structurally unrelated sirtuin family can mediate mono-ADP-ribosylation (Feijs et al. 2013). In addition, ADP-ribose may be attached to proteins by nonenzymatic means (McDonald and Moss 1994). These different reaction types result in a wide spectrum of amino acid specificities that complicate the analysis of their products.

The aim of this review is to provide an overview of current methods for the identification and analysis of ADP-ribosylated proteins, beginning with the characterization of the products of *in vitro* ADP-ribosylation reactions using modified versions of NAD⁺ as a substrate, and continuing to recent advances in the identification and analysis of endogenously modified ADP-ribosylated proteins and of the respective modification sites.

2 Strategies for the Identification of ADP-Ribosylated Proteins

2.1 *Using Modified NAD⁺ to Identify ADP-Ribosylated Proteins in In vitro Reactions*

2.1.1 Use of Radiolabeled NAD⁺

The use of radioactive NAD⁺ as a substrate was the first, and for a long time the only means to visualize ADP-ribosylated proteins. The first ADP-ribosylation reaction ever observed was detected following the incubation of nuclear extracts from liver cells with radioactive ATP in the presence of nicotinamide mononucleotide (Chambon et al. 1963). However, in this case the modified high-molecular weight substrate resulting from the reaction was not correctly identified as polymeric ADP-ribose, but at first misinterpreted to be polyadenylic acid. Only some years later was it realized that the radioactive ATP might have been converted to NAD⁺ by NAD⁺ pyrophosphorylase present in the nuclear extract. Similar experiments using radiolabeled NAD⁺ that had been synthesized prior to incubation were performed almost simultaneously by several groups, and led to the realization that NAD⁺ and not ATP was the immediate substrate and to the identification of polymeric ADP-ribose as the product of the reaction (Chambon et al. 1966; Fujimura et al. 1967; Nishizuka et al. 1967). The radioactive NAD⁺ species developed for these experiments subsequently were used by Honjo et al. to discover ADP-ribosylation as the mechanism causing the inactivation of elongation factor 2 by diphtheria toxin (Honjo et al. 1968). By incubating target and toxin with NAD⁺ carrying radiolabels at different positions within the molecule, these authors found that NAD⁺ labeled at any position outside of nicotinamide caused incorporation of radioactivity into the target protein.

This brief historic introduction may serve to illustrate one of the pitfalls that may be associated with the use of radioactive (or otherwise modified) metabolites to visualize post-translational modifications. The detection of radiolabel does not give any information about whether and how the radioactive substrate has been processed before incorporation into the protein. We will return to this below. However, the use of radioactive NAD⁺ has many advantages that make it a method of choice for the visualization of ADP-ribosylation reactions in many experimental contexts. First, enzymes are usually indifferent to isotope exchanges within their substrates, so radioactive NAD⁺ may be expected to faithfully mimic the behavior of unmodified NAD⁺. Radioactively labeled proteins can be visualized directly with a high sensitivity in many detection systems, such as SDS-PAGE or gel chromatography, that give biochemical information such as molecular size about the target protein.

The incorporation of radioactive ADP-ribose is less useful for the identification of ADP-ribosylation in the context of living cells or for the affinity purification of ADP-ribosylated substrates. In the latter case, it can be used as a tracer to follow the

progress of modified proteins during the purification procedure, but, in contrast to other forms of modified NAD⁺ discussed below, by itself does not provide any mechanism facilitating enrichment.

Radioactive NAD⁺ is commercially readily available in various species differing in the isotopes used for labeling and the positions where the label is incorporated. Most applications use NAD⁺ carrying a radioactive phosphorus atom within the diphosphate bridge at the position proximal to the adenosine moiety ([α -³²P]-NAD⁺). However, NAD⁺ species labeled at other positions are also available and may be useful for certain applications. Especially, NAD⁺ labeled by ¹⁴C within the nicotinamide moiety has been used to differentiate genuine ADP-ribosylation from the formation of unconventional adducts of NAD⁺ with target proteins (McDonald and Moss 1993).

2.1.2 Use of Nonradioactive NAD⁺ Derivatives

Several modifications have been incorporated into the adenosyl moiety of NAD⁺ with the aim of introducing detectable chemical “tags” into ADP-ribosylated target proteins. This approach offers several advantages over the use of radioactivity to label NAD⁺. These tags are recognized by highly specific ligands such as antibodies or streptavidin that can be conjugated to fluorochromes, thus permitting the visualization of ADP-ribosylated proteins by immunofluorescence. In addition, this approach allows ADP-ribosylation reactions occurring on cell surfaces to be followed by flow cytometry (Krebs et al. 2003).

Zhang and Snyder conjugated NAD⁺ to biotin, and reported that biotinylated NAD⁺ could be used by diphtheria toxin to modify elongation factor 2, and by an unknown endogenous nitric oxide-inducible ADP-ribosyltransferase to modify GAPDH (Zhang and Snyder 1993). However, this latter case was later found not to be due to genuine ADP-ribosylation, but to represent unconventional covalent attachments of either NAD⁺ or ADP-ribosyl to GAPDH (McDonald and Moss 1993). In a similar fashion, Takei and colleagues used digoxigenin to label NAD⁺ and showed that the resulting DIG-NAD was incorporated by pertussis toxin into its target protein G_{iα} (Takei et al. 1994). The modified protein was then visualized by immunofluorescence microscopy using anti-digoxigenin antibodies, and found to be localized in the plasma membrane and the nucleus. However, it is important to note that other authors have contested the validity of using biotinylated or digoxigenin-conjugated NAD⁺ as surrogate substrates for ADP-ribosyltransferases. Klebl et al. found that the pattern of target proteins labeled by either pertussis toxin or arginine-specific ADP-ribosyltransferase from skeletal muscle was different when using biotinylated or digoxigenin-conjugated NAD⁺ as compared to that obtained using [³²P]-NAD⁺ (Klebl et al. 1997).

An interesting alternative to the above-mentioned chemical modifications is the introduction of an etheno group into the adenine moiety of NAD⁺. The NAD⁺-analog 1,N6-etheno-NAD⁺ (etheno-NAD⁺) is readily accepted as a substrate for ADP-ribosylation by a broad spectrum of bacterial and eukaryotic ADP-ribosyltransferases

(Armstrong and Merrill 2001; Davis et al. 1998; Giovane et al. 1985; Hingorani and Ho 1988; Klebl and Pette 1996; Krebs et al. 2003; Lowery and Ludden 1988). An antibody (1G4) is available that specifically recognizes 1,N6-ethenoadenosine. This originally had been developed to detect ethenoadenosine as an adduct in DNA resulting from the exposure of workers to vinyl chloride (Young and Santella 1988). The 1G4 antibody detects etheno-ADP-ribose incorporated into both poly- and mono-ADP-ribosylated proteins, and can be used in a variety of assay systems including Western blot and immunofluorescence (Bannas et al. 2005; Davis et al. 1998; Krebs et al. 2003; Liao and Puro 2006). Importantly, fluorochrome-conjugated 1G4 antibody has been used as a flow cytometric assay to monitor cell surface ADP-ribosyltransferase activity (Grahner et al. 2002; Krebs et al. 2003; Scheuplein et al. 2009; Seman et al. 2003). Recently, 1G4 coupled to superparamagnetic iron oxide (SPIO) particles has been used to detect cells carrying etheno-ADP-ribosylated proteins in vivo by magnetic resonance imaging (Bannas et al. 2010).

2.2 Determining the Specificity of ADP-Ribosylation Sites

ADP-ribosyltransferases have been divided into the two families of ARTC and ARTD enzymes (Hottiger et al. 2010). While all molecularly identified mammalian ARTC transferases are specific for arginine as the acceptor residue (Seman et al. 2004), mammalian ARTD transferases preferentially seem to modify lysine, glutamic or aspartic acid, or serine/threonine residues (Feijs et al. 2013). ADP-ribosylation on cysteine and asparagine residues has also been detected biochemically, although the responsible enzymes have not been molecularly defined.

The stability of the ADP-ribosyl-protein bond in different chemical environments can help to elucidate the amino acid linkage of the modification (Cervantes-Laurean et al. 1993; Feijs et al. 2013). The O-glycosidic linkage to acidic residues is more sensitive to neutral hydroxylamine than the N-glycosidic bond to arginine or lysine (Moss et al. 1983). ADP-ribosyl bonds to cysteine are uniquely sensitive to mercuric chloride (Meyer et al. 1988). However, while the enzymatic ADP-ribosyl-cysteine linkage catalyzed by pertussis toxin is resistant to neutral hydroxylamine, the nonenzymatic covalent attachment of free ADP-ribose to cysteine is sensitive to both mercuric chloride and hydroxylamine (McDonald and Moss 1994; McDonald et al. 1992).

2.3 Detection of Endogenously ADP-Ribosylated Proteins

While many tools are available to visualize and identify the products of in vitro ADP-ribosylation reactions via the use of labeled analogs of NAD⁺, it is much more difficult to detect ADP-ribosylated proteins that have been endogenously modified by living cells using their own unlabeled pool of NAD⁺. This section outlines some of the approaches that have been taken to achieve this aim.

2.3.1 Anti-ADP-Ribosyl Antibodies

Already early in the history of ADP-ribosylation several groups made antibodies designed to detect endogenously ADP-ribosylated proteins. Meyer and Hilz immunized rabbits with a nonhydrolyzable ADP-ribose analog or ADP-ribose coupled to bovine serum albumin, and then purified the resulting serum on an affinity column containing ADP-ribosyl-guanidinobutyrate, formed by ADP-ribosylating guanidinobutyrate by cholera toxin. The resulting serum recognized ADP-ribosylated elongation factor 2 in immunoblots and revealed the ADP-ribosylation of nuclear proteins in response to chemical stress by immunofluorescence (Meyer and Hilz 1986). Spiegel and co-workers immunized with ADP-ribose chemically coupled to carrier proteins. The resulting serum recognized G-proteins modified by pertussis toxin and ADP-ribosylated elongation factor 2 on immunoblots (Eide et al. 1986). McMahon and co-workers immunized rabbits with ADP-ribosylated polyarginine and used the resulting serum to detect arginine-ADP-ribosylated proteins in subcellular fractions of quail cardiac muscle by immunoblot (Schwab et al. 2000). More recently, the group of Tsuchiya raised an antiserum to ADP-ribosylated histones, and purified anti-ADP-ribosyl antibodies on an affinity column containing ADP-ribosylated protamine. The resulting serum was used in immunoblot and immunofluorescence to detect ADP-ribosylation on lymphocytes and in skeletal muscle (Osago et al. 2008). Although these attempts have created valuable tools for the detection of endogenously ADP-ribosylated proteins, they have not gained wide distribution. To date, no monoclonal antibody recognizing ADP-ribosyl-arginine or any other ADP-ribosyl linkage is available.

2.3.2 Recombinant Macro Domains as Sensors for ADP-Ribosylated Proteins

Recently, macro domains contained in some intracellular proteins have been identified as binding domains for polymeric and monomeric ADP-ribose (Karras et al. 2005). This property has been exploited to precipitate ADP-ribosylated proteins from extracts of CHO and HL-60 cells using recombinant macro domains from human ARTD9 and the archaeal protein Afl521 (Dani et al. 2009). Subsequent analysis of the precipitated proteins by mass spectrometry revealed several known target proteins of intracellular ADP-ribosylation (see Sect. 3).

2.3.3 Antibodies Against Epitopes Sensitive to ADP-Ribosylation on Target Proteins

In selected cases, antibodies that recognize target proteins of ADP-ribosylation at their modification site have been used to monitor the state of endogenous ADP-ribosylation of the respective protein. For example, integrin α_L/β_2 (LFA-1, CD11a/CD18) can be ADP-ribosylated on both of its two chains. ADP-ribosylation of

LFA-1 interfered with the binding of some antibodies (i.e., mAb 2D7 that recognizes CD11a and mAb C71/16 that recognizes CD18), while the binding of other antibodies was not affected [Nemoto 1996]. Similarly, recognition of CD8 by mAb YTS156.7.7, but not by mAb H35–17.2, was blocked when murine T cells were exposed to extracellular NAD^+ , suggesting that binding of YTS156.7.7 was sensitive to the ADP-ribosylation of its cognate epitope. Further experiments revealed that NAD^+ released from lymph node cells during cell preparation was sufficient to mediate the ADP-ribosylation of CD8 (Lischke et al. 2013).

3 Strategies for the Enrichment of ADP-Ribosylated Proteins

Some of the procedures described above for the detection of ADP-ribosylated proteins can also be used for their specific enrichment in pulldown experiments, either after the introduction of modified NAD^+ in vitro or by the use of antibodies or macro domains for the binding of endogenously ADP-ribosylated proteins.

3.1 *Use of Nonradioactive NAD^+ Derivatives in Pulldown Experiments*

The NAD^+ derivatives described in Sect. 2.1.2 can be used to purify or enrich ADP-ribosylated proteins by affinity chromatography. As a proof of principle, Zhang and Snyder showed that diphtheria toxin could utilize biotinylated NAD^+ as a substrate to incorporate biotin-ADP-ribose into EF2, and used streptavidin beads to isolate the modified protein from cell extracts. They then used the same methodology to purify an unknown protein that was ADP-ribosylated in response to nitric oxide, which they then identified as glyceraldehyde-3-phosphate dehydrogenase (Zhang 1997; Zhang and Snyder 1993). In a similar fashion, we have used the 1G4 antibody specific for ethenoadenosine to purify etheno-ADP-ribosylated membrane proteins from detergent extracts of cells expressing cell surface ARTC2 and incubated with etheno- NAD^+ (our own unpublished observations).

3.2 *Pulldown of Endogenously ADP-Ribosylated Proteins*

Antibodies directed against the ADP-ribosyl moiety (see Sect. 2.3.1) could, in general, be used for the enrichment of ADP-ribosylated proteins in immunoprecipitation experiments. However, the lack of easily available antibodies, particularly monoclonal antibodies, has hampered this approach. Furthermore, single-step immunoprecipitations are prone to a high load of contaminations (Mellacheruvu et al. 2013).

An alternative approach is the pulldown of ADP ribosylated proteins with recombinant macro domains (see Sect. 2.3.2). The use of a recombinant macro domain from the Archaeon *Archaeoglobus fulgidus* for the enrichment of ADP-ribosylated proteins in a pulldown experiment has been described (Dani et al. 2009). The authors used a non-ADP-ribosyl binding mutant of the macro domain for pre-clearing of the extract and in a second step the wild-type macro domain for the specific binding of ADP-ribosylated proteins. Both macro domain constructs carried His₆- or GST-tags for immobilization onto beads. After separation by SDS gel electrophoresis a number of known target proteins for ADP-ribosylation were identified by mass spectrometry (e.g., the heatshock protein GRP78, mitochondrial glutamate dehydrogenase, G protein β subunits, EF 1 α , GAPDH), demonstrating the feasibility of the method for the isolation of proteins ADP-ribosylated at different amino acid side chains.

4 Strategies for the Enrichment of ADP Ribosylated Peptides After Proteolytic Digestion

Post-translational modifications are, in general, not stoichiometric. Furthermore, ADP-ribosylated peptides, just as phosphopeptides, are prone to losses during sample preparation and their mass spectrometric detection poses particular problems (see [Regulation of nitrogenase by reversible mono-ADP-ribosylation](#)). Thus, specific enrichment is advisable at the protein level (see [Photorhabdus luminescens Toxins TccC3 and TccC5: Insecticidal ADP-Ribosyltransferases that Modify Threonine and Glutamine](#)) or after proteolytic digestion at the peptide level.

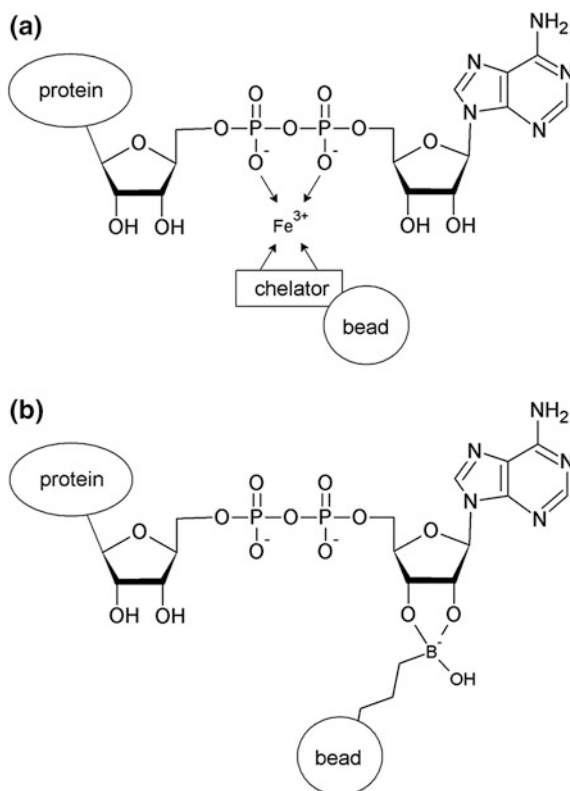
Both the pyrophosphate and the ribosyl moiety of the ADP-ribosyl group can be used for the enrichment of modified peptides. However, both procedures are of limited specificity, since the affinity materials also exhibit an affinity for phosphorylated or glycosylated peptides, respectively.

After any enrichment procedure, the eluted peptide fractions should be desalted by reverse-phase materials (e.g., ZipTips), dried down and redissolved in an appropriate buffer for mass spectrometric analysis (See [Regulation of nitrogenase by reversible mono-ADP-ribosylation](#)).

4.1 Enrichment by Phosphate-Binding Affinity Materials

Affinity enrichment by IMAC or by TiO₂ beads are standard procedures in the analysis of phosphopeptides (Macek et al. 2009; Rosenqvist et al. 2011). In spite of the differences in the chemical structure of the phosphate bonds, similar affinities as for phosphopeptides have been demonstrated for the binding of ADP-ribosylated peptides onto IMAC or TiO₂ beads (Laing et al. 2011). The binding mode of the ADP-ribosyl group to Fe³⁺-IMAC beads is shown schematically in Fig. 1a.

Fig. 1 Enrichment of ADP-ribosylated peptides by binding to solid phase matrices. Schematic showing the interaction of an ADP-ribosylated protein with an IMAC affinity matrix (a) or a boronic acid resin (b)



Enrichment was tested for beads loaded with Fe^{3+} and for magnetic TiO_2 beads using ADP-ribosylated TNF α (double digest with trypsin and Glu-C (synonym: V8 protease)), tryptically digested ADP-ribosylated HNP-1, a well characterized substrate for ADP-ribosylation (Paone et al. 2006), mono- and doubly ADP-ribosylated Kemptide (LRRASLG, a model substrate for both kinases and ADP-ribosyl transferases) and, as a phosphopeptide control, tryptically digested α -casein. All test samples contained an excess of tryptically digested BSA. The results demonstrated clearly that IMAC as well as binding to TiO_2 are suited for the enrichment of ADP-ribosylated peptides. While both methods gave comparable yields for the phosphopeptide controls, Fe^{3+} -IMAC enrichment gave more consistent results and higher yields compared with the enrichment by TiO_2 magnetic beads. However, as often observed for the enrichment of phosphopeptides, the yields varied highly between the different ADP-ribosylated peptides. Therefore, it is not clear whether the superior performance of IMAC can be generalized. For the enrichment of peptides ADP-ribosylated at Asp or Glu side chains the elution conditions have to be modified, since ADP-ribosylated carboxylate side chains are susceptible to hydrolysis at basic pH values (Cervantes-Laurean et al. 1997).

Finally, it should be noted that, since ADP-ribosylated peptides are copurified with phosphopeptides in IMAC and TiO_2 enrichment experiments (as outlined above), ADP-ribosylation sites can be identified in phosphoproteomics data sets (Matic et al. 2012).

4.2 Enrichment by Boronate Affinity Materials

Boronate affinity chromatography is an established method for the isolation of 1,2-cis-diol-containing compounds (Liu and Scouten 2000, 2005). Even though its application for the purification of ADP-ribosylated peptides has been described early on (Okayama et al. 1978), the method has rarely been used. A detailed description is given by Rosenthal and colleagues (Rosenthal et al. 2011). In short, ADP-ribosylated peptides are bound to boronic acid resins at moderately basic conditions and high ionic strength (pH 8.7–8.8, 0.5–1 M NaCl, 0–50 mM MgCl_2). The binding mode of the ADP-ribosyl group to boronic acid beads is shown schematically in Fig. 1b. The bound peptides are eluted at low pH (e.g., 0.1 M acetic acid) or by competition with a diol-containing compound (e.g., 50 mM sorbitol).

5 Identification of Protein ADP-Ribosylation Sites

The identification of protein post-translational modification sites in modern proteome analysis relies almost exclusively on the mass spectrometric analysis after proteolytic digestion. If the ADP-ribosylated protein can be isolated and identified, the detection of a peptide with a mass increment of 541.06 Da can provide a strong hint at the site of a mono-ADP-ribosylation (Margarit et al. 2006). Putative ADP-ribosylation sites can be confirmed by site-directed mutagenesis (Ganesan et al. 1998). Similar to phosphopeptides, ADP-ribosylated peptides have a high affinity for mono- and divalent cations, and even after careful purification, sodium and potassium adducts are usually detected in addition to the protonated species (unpublished observation) in positive mode mass spectrometry. While this effect on the one hand hampers the detection by decreasing the sensitivity, it corroborates, on the other hand, the presence of a phosphate group in the respective peptide. However, the definite proof of an ADP-ribosyl modification and its exact location can only be obtained from the analysis of the fragment pattern in MS/MS mass spectrometric experiments (for a review see (Hengel and Goodlett 2012)).

5.1 Identification by MS/MS Fragment Analysis

5.1.1 Collision-induced Decay

The standard technique for the analysis of peptides by tandem mass spectrometry is CID, (synonymous: collision-activated decay, CAD). In a collision cell filled with an inert gas (mostly argon or helium), analytes are accelerated by an applied voltage. Collision with the inert gas molecules results in the conversion of kinetic into vibrational energy until chemical bonds are broken. The analyte is then identified by its fragmentation pattern. Peptides typically fragment at peptide bonds, while most post-translational modifications are either stable in a CID experiment (e.g., phosphotyrosine, acetylsine) or the modification is eliminated as a neutral leaving group (“neutral loss”, e.g., phosphoric acid from phosphoserine, mono-saccharides from glycopeptides). ADP-ribosylated peptides, however, display a different behavior, which poses a considerable obstacle to their detection and identification: upon fragmentation, ADP-ribosylated peptides release predominantly not the modifying ADP-ribosyl group, but the peptide as a neutral loss, while the ADP-ribose group or its fragments carry the charge(s) (Hengel et al. 2009). These authors have proposed a nomenclature for the fragments of the ADP-ribosyl group that are typically detected upon fragmentation of ADP-ribosylated peptides. These are listed in Table 1. Peptide fragments needed for sequence identification are small or not detectable. The extent of this problem is controversial and may depend on the experimental setting. Upon fragmentation in an ion trap instrument, the neutral loss of the peptide component was found to be pronounced only for smaller peptides, while fragmentation of larger ADP-ribosylated peptides resulted in the detection of both peptide and ADP-ribose fragments (Hengel et al. 2010). By contrast, in a QTOF instrument the fragmentation pattern of all ADP-ribosylated peptides was dominated by fragments of ADP-ribose (Laing et al. 2011).

While the fragmentation pattern of ADP-ribosylated peptides hampers the unambiguous identification of the site of modification, the strong fragment signals of the ADP-ribosyl group can, on the other hand, be used for the sensitive detection of the ADP-ribosylated peptides in LC/MS experiments. In samples of moderate complexity ADP-ribosylated peptides can also be identified by the alternating acquisition of spectra at low and high collision energy in LC/MS experiments. In the spectra recorded at high collision energy, ADP-ribosylated peptides give rise to a series of characteristic fragments (Table 1), the signals of which can be traced in the respective extracted ion chromatograms. In the spectra recorded at low collision energy the candidate peptides can be identified and validated (Laing et al. 2011). This approach is essentially an application of the general approach described for the identification of labile post-translational modifications (Carapito et al. 2009).

Osago and colleagues studied the CID fragmentation of ADP-ribosylated peptides in positive and negative mode in a quadrupol tandem instrument (Osago et al. 2009). In addition to the fragments listed in Table 1 the authors describe a singly charged fragment with $m/z = 582.4$ (negative mode) or $m/z = 584.4$ (positive mode),

Table 1 Fragments detected after CID fragmentation of ADP-ribosylated peptides

Theoret. mass as MH ⁺ [Da]	Assignment	Fragment type ^a
136.06	Adenine	m ₁
250.09	Adenosine-18	m ₃
348.07	Adenosine-monophosphate	m ₆
428.04	Adenosine diphosphate	m ₈

^a Nomenclature according to Hengel et al. (2009)

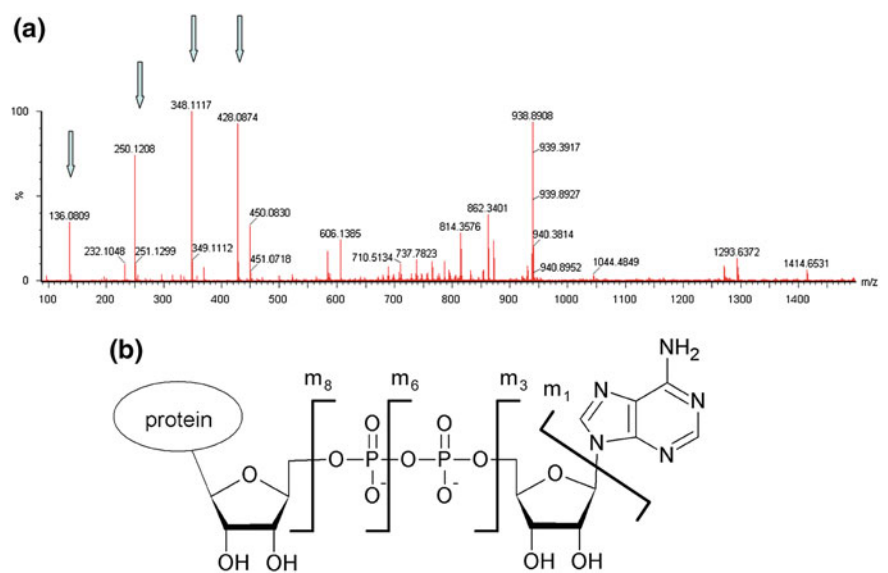


Fig. 2 Fragmentation of ADP-ribosylated peptides in CID fragmentation experiments. **a.** MS/MS spectrum of di-ADP-ribosylated Kemptide after CID fragmentation. Fragments of the ADP-ribosyl groups (listed in Table 1) are marked by *arrows*. Nanospray ESI experiments were performed on a QTOF II instrument (Waters), using 500 fMol Kemptide/ μ l in 60 % MeOH/5 % HCOOH; precursor selection: $m/z = 927.3$ (MH_2^{2+}); for further experimental details see (Laing et al. 2010). **b.** Fragmentation pattern of the ADP-ribosyl group in CID fragmentation experiments

which can be attributed to the carbodiimide derivative of the ADP-ribosyl group, and results from a cleavage within the arginine guanidinium group. This fragment can be used for the identification of ADP-ribosylated peptides in a precursor ion scanning experiment. It should, however, be noted that it can be detected only if arginine is the site of ADP-ribosylation (Fig. 2).

5.1.2 Fragmentation by ECD or ETD

Electron capture dissociation and electron transfer dissociation are fragmentation techniques in which low energy electrons are transferred onto multiply charged peptides either directly (ECD) or chemically mediated by a radical anion (ETD). The radical cation analytes that arise from this process fragment by a mechanism different from CID and give rise to different fragmentation products. Most notably, ECD and ETD lead preferentially to peptide backbone fragmentation while leaving side chain modifications intact. This has been shown for phosphorylations and has been demonstrated to be the case also for ADP-ribosylations in ECD (Hengel et al. 2009) and ETD (Zee and Garcia 2009) experiments. Thus, ECD or ETD can be used in different experimental schemes for the simultaneous identification of the ADP-ribosyl group and the peptide to which it is attached. This has also been shown for peptides, which are ADP-ribosylated at lysine side chains by a nonenzymatic reaction (Fedorova et al. 2010).

In CID experiments, potentially ADP-ribosylated peptides can be identified in precursor ion scanning experiments (Osago et al. 2009), in MS/MS experiments (Hengel et al. 2009), or by data-independent acquisition (Laing et al. 2011). The respective peptide sequence can then unambiguously be determined by ECD or ETD.

5.2 *Identification of ADP-Ribosylation Sites After Enzymatic or Chemical Cleavage of the ADP-Ribosyl Group*

While the removal of the modifying group is mandatory for the analysis of poly(ADP-ribosyl)ated proteins due to the complex structure of the modification, this may also facilitate the detection of mono-ADP-ribosylation sites. Any treatment should reduce the complexity of the modification in order to make it more manageable, while leaving a tag on the modified amino acid side chain to unambiguously identify the site of modification.

It has long been known that poly(ADP-ribose) can be digested by snake venom phosphodiesterase (Matsubara et al. 1970). The pyrophosphate bond between the ADP-ribosyl-phosphate groups is cleaved by the phosphodiesterase, leaving a phosphoribosyl group attached to the amino acid side chain.

Recently the use of this reaction for the identification of auto-ADP-ribosylation sites in PARP1 (ARTD1) has been described and the applicability for both mono- and poly-ADP-ribosylated peptides has been demonstrated (Chapman et al. 2013). Phosphoribosylated peptides exhibit properties comparable to phosphopeptides insofar as they can be enriched by standard phosphoproteomics procedures (e.g., IMAC) and show similar fragmentation characteristics upon CID in mass spectrometric experiments, providing, in contrast to ADP-ribosylated peptides, ample fragment signals for peptide identification.

For the analysis of ADP-ribosylated peptides modified at Asp or Glu side chains, the ADP-ribosyl group can be cleaved by treatment with hydroxylamine (Zhang et al. 2013), leaving a hydroxamic acid derivative at the modified side chain. The mass increment of + 15 Da serves as a tag for ADP-ribosylation. ADP-ribosylated Arg is cleaved only slowly (Moss et al. 1983) and all other ADP-ribosylated amino acid side chains are refractory to hydroxylamine treatment (Cervantes-Laurean et al. 1997).

6 Conclusion

ADP-ribosylated proteins can be identified after in vitro labeling with radioactively labeled or chemically modified ADP-ribosyl derivatives. Endogenously ADP-ribosylated proteins can be identified with anti-ADP-ribosyl antibodies or ADP-ribosyl binding macro domains. Some of these techniques can also be used for enrichment.

After proteolytic cleavage, ADP-ribosylated peptides can be enriched by binding to immobilized metal ion (IMAC) beads or to boronic acid beads.

In tandem mass spectrometric experiments, fragmentation by CID typically results in characteristic fragments of the ADP-ribosyl moiety, while only weak signals of peptide fragments are detected. Thus, CID experiments are useful for the detection of an ADP-ribosyl modification, but often do not allow the identification of the peptide to which it is attached. This problem can be overcome by alternative fragmentation techniques (ECD or ETD). Another option is the phosphodiesterase treatment of the sample, converting ADP-ribosylated peptides into phosphoribosylated peptides (which have properties comparable to phosphopeptides, or, in the case of ADP-ribosylated Asp or Glu side chains, the treatment with hydroxylamine, converting ADP-ribosyl groups into hydroxamic acid derivatives (which are stable upon CID fragmentation)).

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